# CONTROL FOR UNIFORMITY OF SYNTHETIC PEPTIDES: QUANTITATIVE EVALUATION OF THE EDMAN DEGRADATION OF SUPPORT–BOUND HUMAN INSULIN B 22–30 SYNTHETIZED BY THE MERRIFIELD METHOD

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## 1. Introduction

The demand for completion of all transformations in the Merrifield synthesis of peptides is widely accepted as the categorical imperative of the method, which in the future aims at well defined synthetic proteins [2]. Continually performed analytical monitoring of all steps [3] is essential, since deviations of <1% from quantitative reactions seriously endanger the success of an extended peptide synthesis (> 10 amino acid residues) on polymer support by formation of false sequences [4].

Nevertheless, besides our methodical and analytical improvements [5] of the Merrifield technique [6] there remains the question for final uniformity of the peptide sequence synthetized on insoluble polymers. To answer this question we modified the Laursen method [7] of Edman degradation of peptides bound to insoluble supports for direct formation of pure phenylthiohydantoines (PTHs) [8], which, without chemical manipulations, are directly separated and quantitatively identified by high pressure liquid chromatography (HPLC) [9,10]. Independently from the sometimes misleading results from amino acid analysis after total hydrolysis of support-bound peptides our modification permits to detect quantitatively < 0.5% of sequential inhomogeneities in peptides synthetized on insoluble polymers and still attached to the support.

* Part of diploma work (1975) Heidelberg, [1].

## 2. Materials

Peptide Synthesizer (Schwarz/Man, Orangeburg, N.Y., USA) equipped with the centrifugal reactor [11] (LKB Instruments, Munich, FRG) Synthesis control system [3] (SCS): Centrifugal reactor, Uvicord III (LKB Instruments, Munich), 3-Channel recorder with automatic 15-times scale expansion and integrator (Linseis, Selb, FRG). UV spectrophotometer DB-G and Amino acid analyzer Unichrom C (Beckman, Munich), Sequemat (Sequemat Inc., Watertown, Mass., USA), modified for direct PTH conversion [8]. HPLC-System (DuPont 830, Friedberg, FRG); Spectroflow SF770 (Schoeffel, Trappenkamp, FRG) technical data see preceding paper [9]; Ddz**-hydrazide and some of the Ddz-amino acids were purchased from C. H. Boehringer Sohn (Ingelheim, FRG) and used analytically pure. All reagents and solvents both for solid phase peptide synthesis and Edman degradation were purified as published [5,8].

## 3. Methods

Starting from 1 g of polystyrene gel (< 0.5% DVB) esterified [12] with 0.250 mmol Ddz -Thr ($Bu^t$), the Merrifield synthesis of sequence B 22-30 (human

** *Abreviations*: Ddz-α,α-Dimethyl-3,5-dimethoxybenzyloxycarbonyl-([12], acid-sensitive protecting group); DVB: divinylbenzene (cross-linking agent); DCC: dicyclohexylcarbodiimide; NPA: 3-nitrophthalic anhydride (blocking agent for suppression of failure sequences).



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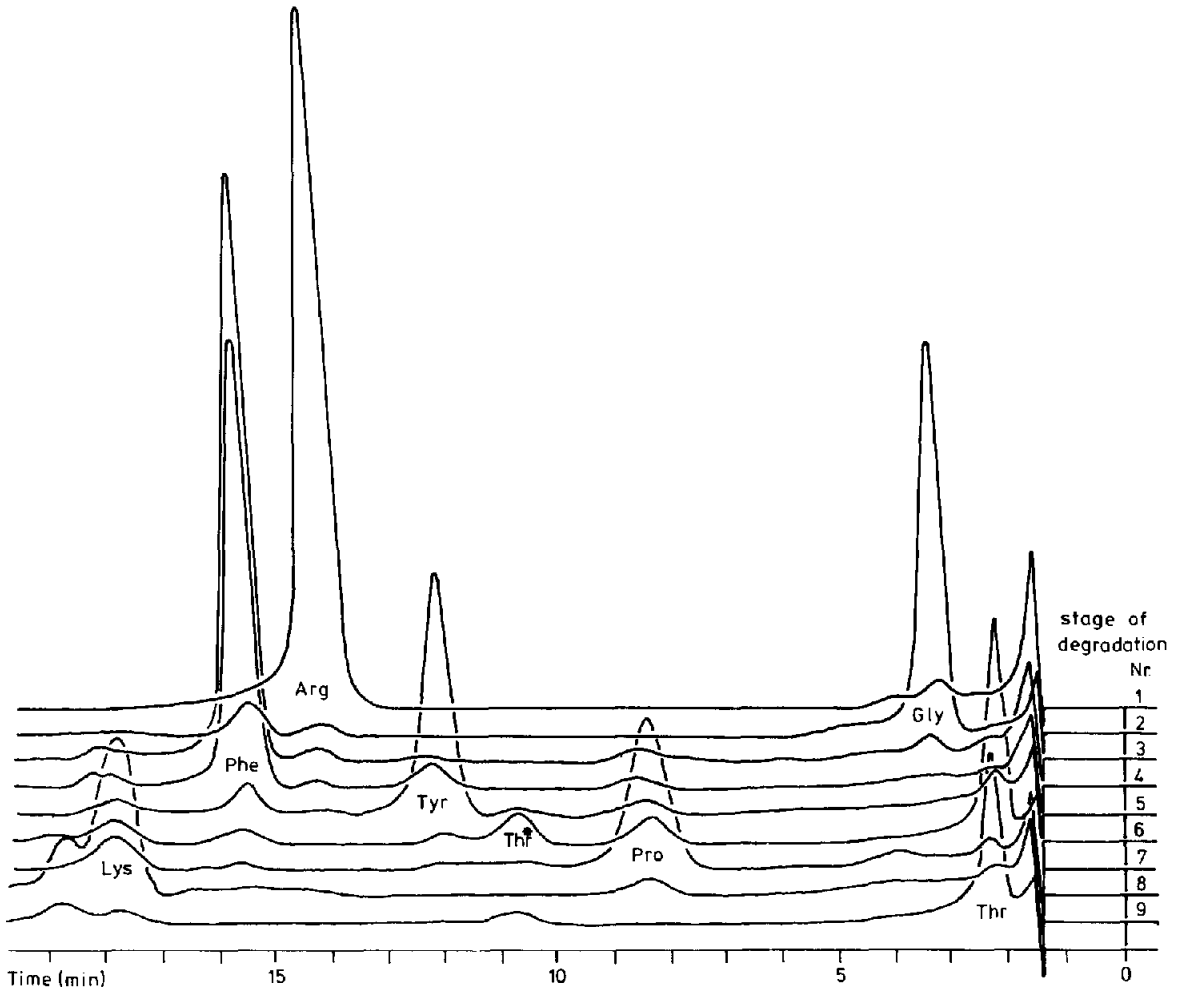


Fig.1. HPL chromatogram of the PTH pattern from solid phase degradation of the nonapeptide B22-30 (Human Insulin) synthetized on solid phase. (Thr*: PTH-dehydro-Thr)

insulin) was performed in the centrifugal reactor utilizing three times a five-fold excess of Ddz-amino acid and DCC in 90 ml dichloromethane (total volume) at each stage of the synthesis. All operations, including NPA treatment [14], were programmed as published [5] and monitored by SCS [3]. Incorporation of Ddz-amino acids (mmol/g starting polymer) [5] during hexapeptide synthesis: 0.250 Thr($Bu^t$); 0.251 Lys(Z); 0.250 Pro; 0.241 Thr($Bu^t$); 0.260 Tyr ($Bu^t$); 0.245 Phe.

40 mg of gel polymer supporting the hexapeptide (I) was taken out of the reactor for intermediate control of the synthesis by solid phase Edman degradation. Finally, the synthesis yielded 1.32 g† of polymer supported fully protected nonapeptide. Ddz–Arg(Tos)–Gly–Phe–Phe–Tyr($Bu^t$)–Thr($Bu^t$)–Pro–Lys(Z)–Thr($Bu^t$)–*O*–polystyrene gel phase (II). 10 mg of protected nonapeptide on polymer were hydrolyzed in a sealed vial with conc. HCl/propionic acid 1:1 (v,v) for 15 min at 160°C [15] and analyzed (theor. values in brackets) Arg 1.01 (1); Gly 1.00 (1); Phe 1.82 (2); Thr 1.82 (2); Pro 1.17 (1); Tyr 0.73 (1); Lys 1.29 (1). 7.25 mg of I and 8.70 mg of

† Not corrected for mechanical losses from tests and emptying of the reactor.

Table 1
Quantitative evaluation of the HPLC results from degradation of the nonapeptide B22-30 (Human Insulin) synthetized on solid phase

Degradation of Ddz–Arg(Tos)–Gly–Phe–Phe–Tyr($Bu^t$)–Thr($Bu^t$)–Pro–Lys(Z) Thr($Bu^t$)–$OCH_2$-polystyrene (8.7 mg)

| | | Stages of degradation no. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| PTH | K' [a] | 1 [b] | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| –Arg(Tos) | 10.4 | m 173 | i 1.4 1.2 | i 0.8 0.8 | | | | | | |
| –Gly[c] | 1.46 | s 2.6 1.5 | m 141 | i 2.2 2.1 | | | | | | |
| –Phe | 11.4 | | s 4.9 3.5 | m 107 | m 83 | i 5.2 7.9 | i 0.6 1.2 | | | |
| –Thr | 8.34 | | | | s 6.5 7.8 | m 66 | i 0.5 1.0 | | | |
| –Thr[d] | 0.65 | | | | s 0.3 0.4 | s 6.0 9.1 | m 50 | s,i 1.6 3.4 | s,i 0.5 1.2 | m 26 |
| –Pro | 5.58 | | | s 0.6 0.6 | s 0.6 0.7 | s 2.1 3.2 | s 6.9 13.8 | m 47 | i 1.1 3.0 | |
| –Lys(PTC) | 12.42 | | | s 0.4 0.4 | s 0.8 1.0 | s 0.7 1.1 | s 3.5 7.0 | s 8.5 18.1 | m 40 | i 0.2 0.8 |
| Total [e] | | 175.6 | 147.3 | 111 | 91.2 | 80.0 | 61.5 | 57.1 | 41.6 | 26.2 |
| Efficiency [f] | | 70[g] | 84 | 75 | 83 | 88 | 77 | 93 | 73 | (63)[h] |

[a] K' = capacity factor, $\frac{t_1 - t_0}{t_0}$, standarized retention time [9].

[b] On each stage of degradation the first column symbolizes the type of PTH: m, from main sequence; s, from sub-sequence; i, from incomplete degradation on preceding stage. The second column gives the yield, μmol PTH/g polymer supported nonapeptide. The third column shows the % ratio of PTH yield from s- and i-degradations to main PTH.

[c] corrected for incomplete conversion (23%).

[d] corrected for 30% formation of PTH-dehydro-Thr.

[e] μmol PTHs/g polymer supported nonapeptide.

[f] % ratio of specific total yield to the preceding one.

[g] with respect to starting load on polymer (250 μM/g)

[h] Not typical for the average efficiency (80%), because of benzyl ester type of C-terminal peptide bond to polymer.

II for control of sequential uniformity were degraded by the Laursen method [7] as described in the preceding papers [8,9]. To charge the column of the Sequemat apparatus with stationary solid phase [5], samples I and II, respectively, were mixed with glass beads (960 mg each; 200–400 mesh). The operations for degradation including direct conversion to PTH-amino acids were programmed as described [8]. The HPLC analyses of the PTHs obtained were performed as demonstrated in the preceding communication [9].

## 4. Results and discussion

The HPLC elution diagrams only from degradation of II are summarized and evaluated in fig.1 and table 1, respectively, to show the main essentials. Except for the content of 1.5% of the octapeptide B 23-30 (not acylated by Ddz-Arg(Tos)$^{B22}$ in the last step of the Merrifield synthesis) the final nonapeptide is not contaminated by any other truncated [4] sequence (see first stage of degradation, fig.1). Besides the main sequence, an octapeptide that lacks Gly$^{B23}$ can be detected at a level of 2% (failure [4] sequence; see second and subsequent stages of degradation, fig.1). These two subsequences characterize a synthetic inhomogenity of 3.5 ± 0.5% [9] of the desired nonapeptide, which therefore is of 96.5% uniformity. The result fits very well with data from measurement of incorporation of Ddz-amino acids (98% Ddz-Phe$^{B25}$) and from amino acid analysis (see Methods); the continuous SCS showed no significant drop in the recording of the transformations during Merrifield synthesis.

Shorter sequences degraded (stage 3–8) most probably are the consequence of peptide fission by trifluoroacetic acid action (45°C) during degradation, accelerated by anchimeric affects from deprotected hydroxyl side functions on Tyr$^{B26}$, Thr$^{B27}$, and Thr$^{B30}$. In addition we measured an average cleavage of 10% of the peptide ester bond to its support by trifluoroacetic acid per stage of degradation. This partly explains the 20% lowered efficiency of the degradation/stage, which is effected mainly by insufficient solvation of the insolubilized peptide on polymer support.

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